Neokyotorphin and neokyotorphin (1–4): secretion by erythrocytes and regulation of tumor cell growth

Elena Yu. Blishchenko*, Olga A. Mernenko, Oleg N. Yatskin, Rustam H. Ziganshin, Marina M. Philippova, Andrei A. Karelin, Vadim T. Ivanov

Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry Russian Academy of Sciences, ul. Miklukho-Maklaya, 16/10, 117871 Moscow, GSP-7, Russia

Received 16 June 1997; revised version received 15 July 1997

Abstract Human erythrocytes release neokyotorphin, the 137–141 fragment of hemoglobin α-chain into the supernatant of red blood cells primary culture. However, the neokyotorphin fragment 1–4 that is formed together with neokyotorphin inside the red blood cells and in various tissues is not found in the supernatant. Both neokyotorphin and its 1–4 fragment were shown to stimulate proliferation of L929 tumor cells.

Key words: Neokyotorphin; Erythrocytes primary culture; Tumor cell; Proliferation

1. Introduction

Neokyotorphin (NKT) is a well-studied biologically active peptide. This peptide is active in a number of biological tests, in particular, NKT exhibits naloxone-dependent analgesic activity [1], regulates hibernation [2] and increases potential-dependent Ca^{2+} current of frog cardiocytes [3].

Both neokyotorphin (NKT) and des-Arg-neokyotorphin (des-Arg-NKT, NKT (1–4)) were identified earlier in lung, heart and brain tissues [1,2,4] as well as in the erythrocyte lysate [5]. The content of NKT in erythrocytes is significantly lower than in lung (7-fold) and heart (2-fold) tissues and is comparable with the content in brain extracts, while the level of des-Arg-NKT in the above mentioned extracts was similar to that in erythrocyte lysate [4]. NKT/des-Arg-NKT ratio differed significantly in the examined tissues (from 113:1 in heart to 16:1 in lung), being 1:1 in the erythrocyte lysate [4]. The differences in NKT/des-Arg-NKT ratio allowed to suggest that accumulation and proteolysis of NKT in tissues could be of functional significance in vivo. Bearing in mind that des-Arg-NKT shows cytolytic activity in tumor cell lines, that NKT itself is inactive in the same test system [4] and that NKT was detected in human carcinoma tissue [6] we assumed that these peptides could participate in regulation of tumor development. Therefore we studied the release of NKT and des-Arg-NKT from the erythrocytes and the influence of both peptides on proliferation of tumor cells.

2. Materials and methods

2.1. Peptide synthesis

Synthesis of NKT and des-Arg-NKT was carried out as described in [2].

2.2. Preparation of human erythrocytes

Peripheral venous blood of healthy volunteers was obtained from 10 healthy volunteers by venipuncture after confirmation of their health status by Research Hematological Centre, Russian Academy of Medical Sciences.

Blood samples (25 ml) were placed into the tubes containing citrate buffer (to final citrate concentration of 0.25%). The cells were separated from plasma by centrifugation at 1000 rpm for 15 min at 0°C. The obtained pellet (10–12 millions of cells, 99% of erythrocytes) was washed four times by PBS (0.025 M NaH_2PO_4 containing 0.1 M NaCl, pH 7.2).

2.3. Supernatant of the primary culture of erythrocytes

Five ml of washed cells were incubated with 20 ml of PBS (2.5×10^9 cells/ml) in culture flacks for 80 min at 37°C. After 20, 40 and 80 min of incubation, 8 ml of cell culture medium were centrifuged in conditions described above. The obtained supernatants were lyophilised.

2.3.1. RP-HPLC separation. The lyophilisates were dissolved in 250 μl of 0.1% solution of TFA in water and subjected to separation on Nucleosil 120/7μ C_{18} column (10.0×250 mm) (Masherey-Nagel, Germany), equilibrated with buffer A (0.1% TFA in water). The elution was performed for 50 min by linear gradient of acetonitrile from 0 to 30% of buffer B (0.1% TFA, 80% acetonitrile solution in water) at 2.0 ml/min. The detection was carried out at 226 nm. Absorption range is given in mV (1800 mV = 2.56 AU). Reproducibility of the elution profiles was examined in 7 experiments. Standard deviations are given in Fig. 2.

2.4. Amino acid sequences

Amino acid sequences of isolated peptides were determined in the gas-phase sequencer Applied Biosystems 447A (Foster City, USA). Estimation of the peptide content was made from the sequencing data.

2.5. Cell culturing

L929 cells (transformed murine fibroblasts) were cultured as described in [4]. Briefly, the cells were generated in DMEM medium enriched with 10% foetal calf serum (Gibco BRL) and supplemented with 2 mM of glutamine, 100 U/ml of penicillin and 100 mg/ml of streptomycin (Gibco BRL).

2.6. Proliferation assay

L929 cells (5×10^4/well) were placed into 96 well plates (Flow Lab.) and co-incubated with 100 ml of culture medium containing different concentrations of test substances. The peptides were added to the target cells at the beginning of the experiment and after 24 h of incubation. Untreated cells co-incubated with culture medium were used as a control. The cells were incubated for 42 h at 37°C, washed 3-fold by buffer A, fixed by methanol for 10 min, dried and stained by crystal violet solution in 20% ethanol for 1 h. Optical density in the wells containing crystal violet stained cells was determined by Multiscan MCC/340 spectrophotometer at 540 nm [7]. Influence of the test substances on proliferation of L929 cells was determined according to:

$$\text{Change of total cell number (\%)} = \frac{OD_{540}\text{ in the sample} - OD_{540}\text{ in the control}}{OD_{540}\text{ in the control}} \times 100\% \quad (1)$$

*Corresponding author.

PII S0014-5793(97)00991-5

Influence of the test substances on proliferative rate of target cells was calculated as described in [8]:

Change of proliferative rate (%) =

$$\frac{OD_{540} \text{ in the sample} - OD_{540} \text{ in the control}}{OD_{540} \text{ in the control} - OD_{540} \text{ for initial cell number}} \times 100\% \quad (2)$$

OD_{540} for initial cell number was determined before beginning of the incubation procedure. To estimate the background absorption for each plate OD_{540} of the wells containing culture medium was determined. The reproducibility of results was tested in 5 separate experiments. The results of evaluation of statistical significance of the obtained values and corresponding standard deviations are given in Fig. 3.

3. Results

To examine weather NKT and des-Arg-NKT are released by erythrocytes, human red cells were incubated in phosphate buffer (buffer A) for 20, 40 and 80 min at 37°C. The obtained supernatants were subjected to RP-HPLC separation and the peaks corresponding to NKT and des-Arg-NKT standards were isolated (Fig. 1). Determination of the amino acid sequences of the obtained substances showed that erythrocytes secrete NKT, but not des-Arg-NKT, since the peaks with elution parameters corresponding to des-Arg-NKT standards were due to dipeptide LV (peak 1) and a non-peptide compound (peak 2). Thus, erythrocytes do not release detectable amounts of des-Arg-NKT, the NKT/ des-Arg-NKT ratio in erythrocytes being about 1:1. The results obtained demonstrate the selectivity of the secretion of peptides formed inside erythrocytes. They also speak of the fact that hemolysis of the erythrocytes practically does not take place during the incubation procedure.

Fig. 2 shows the time dependence of NKT release. The content of NKT was estimated from the sequencing data and from the measurement of peak areas by means of Multy-ChromSpectr program (version 2.67). As seen from Fig. 2, reliable level of NKT (about 10 pmol/ml) is detected after 20 min of erythrocyte incubation, and after 1.5 h the content of the peptide increases approximately 1.5-fold. From these data we conclude that the peptide is secreted by the cells from the very beginning of incubation and is accumulated with relatively slow rate in the culture medium.

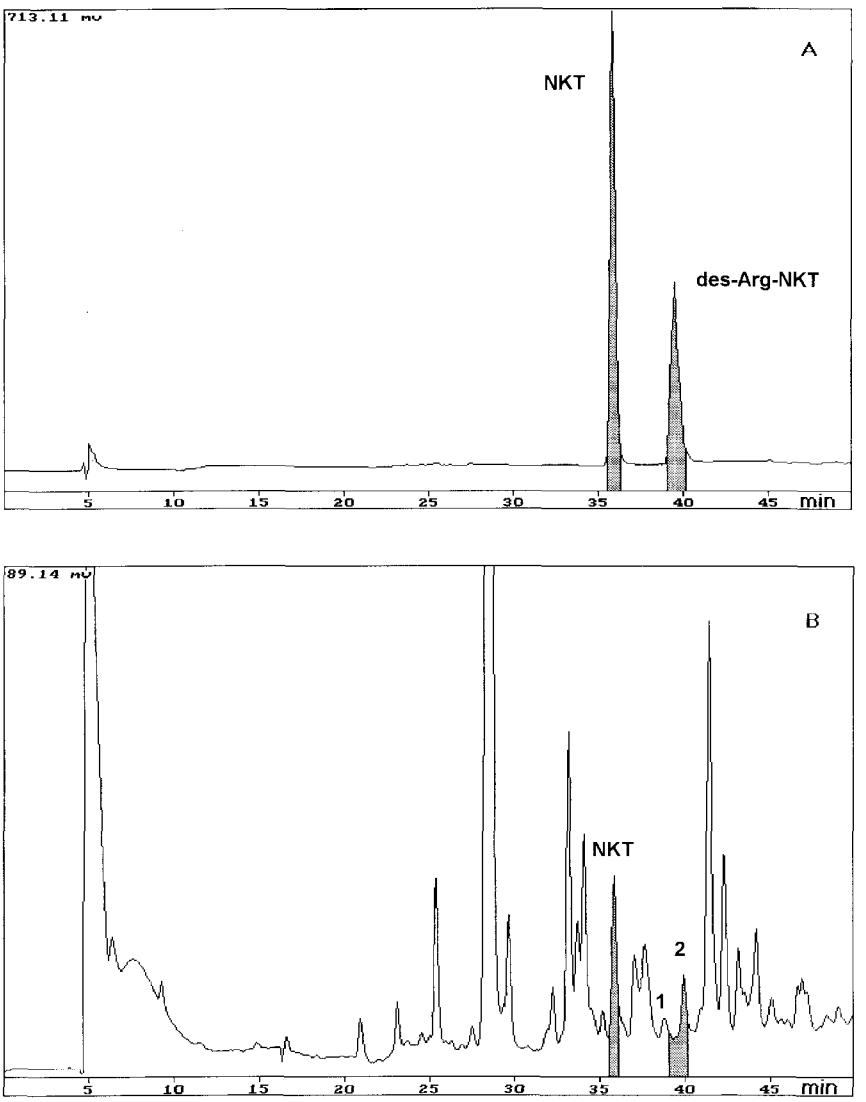

Fig. 1. RP-HPLC of supernatant of primary culture of human erythrocytes. Elution zones of NKT and des-Arg-NKT are shaded.

Des-Arg-NKT was shown earlier to induce cytolysis of human erythroid leukemia (K562) and L929 tumor cells, NKT being inactive in the same conditions [4]. The test applied allowed to calculate the dead/total cell ratio but not the influence of the test substances on proliferation of target cells [9]. In this work we examined the influence of both peptides on growth of L929 cells. As seen from Fig. 3, both peptides increased significantly L929 cell number. Obtained results speak of ability of both peptides to stimulated proliferation of tumor cells. Proliferative activity of the peptides was calculated using Eq. 2: 10^{-6} M of NKT caused approximately 1.5-fold increase of proliferative rate of L929 cells ($56 \pm 8\%$), while des-Arg-NKT showed lesser values of proliferative activity ($37 \pm 6\%$). The detected difference might be due to lysis of a restricted population of target cells by des-Arg-NKT [5]. Thus, NKT exhibits only proliferative activity, while its 1–4 fragment has both growth regulatory and cytotoxic effects [5] in tumor cells.

4. Discussion

Differences in the content of NKT and des-Arg-NKT in tissue extracts could be due both to differences in the secretion rate of the peptides from the erythrocytes and to differences in the proteolytic activity of tissue-specific proteinases [4]. Since erythrocytes were shown recently to release biologically active hemoglobin fragments [10], we have examined the possibility of NKT and des-Arg-NKT secretion by these cells.

It follows from the obtained results that the presence of NKT in different tissues is, at least partially, due to secretion of this peptide by erythrocytes. At the same time, des-Arg-NKT might be formed from NKT by tissue proteolytic enzymes or released from the destroyed erythrocytes. Thus, we established (i) the selectivity of secretion of the peptides formed inside the erythrocytes, (ii) the difference in the mechanism of generation of hemoglobin-derived peptides in tissues.

As soon as both NKT and des-Arg-NKT increased the number of L929, we cells assumed that both peptides act as growth factors of L929 cells, while the less values of the effect mediated by des-Arg-NKT were due to cytotoxic activity of this peptide.

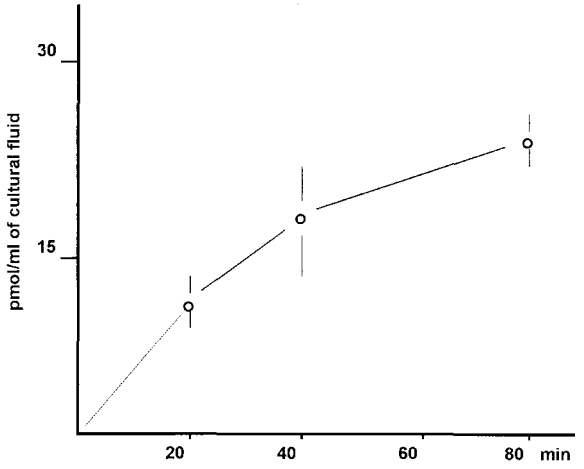

Fig. 2. Time dependence of NKT secretion by human erythrocytes (concentration of the erythrocytes was 2.5×10^9 cells/ml). S.D. is represented by bars.

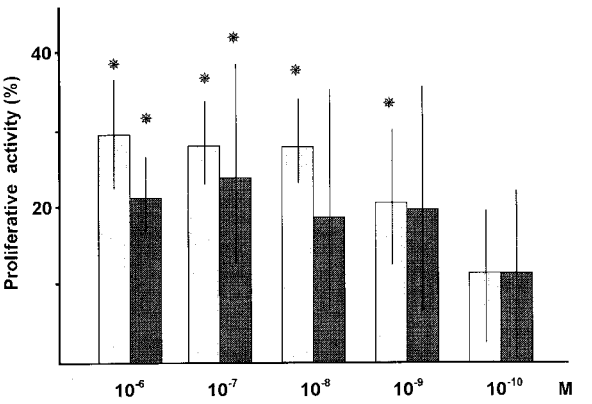

Fig. 3. Proliferative activity of NKT (light) and des-Arg-NKT (dark). S.D. is represented by bars. Statistically significant data ($P < 0.05$) are marked as *.

Des-Arg-NKT was shown earlier to induce several cytolytic processes differing in concentration parameters and DNA fragmentation mechanisms both in L929 and K562 cells [4]. The obtained results allow to suggest that L929 cell subgroups respond differently to the action of des-Arg-NKT, i.e. the peptide increases proliferation rate of the larger part of the cells and kills a restricted portion of the same cell population. The discrete maxima of cytotoxicity are explained by differences in time required for development of respective cytolytic process [10]. Possibly, these maxima reflect differences in the sensitivity of the cells passing through different cell cycle stages to the action of this cytotoxic peptide [11]. Since cytotoxicity of des-Arg-NKT is relatively low, we assume that only one particular stage of the cell cycle is sensitive to the cytotoxic action of the peptide.

As far as the ability to induce both proliferative and cytolytic/cytostatic effects (dependent on tumor line type and on concentration of the test substance) is a characteristic feature of a variety of biologically active peptides [8,12], des-Arg-NKT might also cause effective lysis and, accordingly, decrease of cell number in other tumor cell lines.

The obtained results show that both peptides might participate in regulation of tumor cell growth. In particular, tumor development could be a consequence of enhanced accumulation of NKT due to increased secretion of the peptide or to its reduced degradation rate. In any case, the effects exhibited by NKT and its fragment fall in line with the earlier formulated concept of the role of fragments of functional proteins in maintenance of tissue homeostasis, i.e. in regulation of proliferation, differentiation and death of both normal and tumor cells [9].

Acknowledgements: The study is supported by the Russian State Foundation for Fundamental Research (grant 97-0449197).

References

[1] Takagi, H., Shiomi, H., Fukui, K., Hayashi, K., Kiso, Y. and Kitagawa, K. (1982) Life Sci. 31, 1733–1736.
[2] Ziganshin, R.H., Svieryaev, V.I., Vaskovsky, B.V., Mikhaleva, I.I., Ivanov, V.T., Kokoz, Yu.M., Alekseev, A.E., Korystova, A.F., Sukhova, G.S., Emelyanova, T.G. and Usenko, A.B. (1994) Bioorgan. Khim. 20, 677–702. in Russian
[3] Ziganshin, R.H., Mikhaleva, I.I., Ivanov, V.T., Kokoz, Yu.M., Alekseev, A.E., Korystova, A.F., Mavlyutova, D.A., Emelyanova, T.G. and Akhrimenko, A.K. (1995) In: Peptides. Chemistry,

Structure and Biology (Kaumaya, P.T.P. and Hodges, R.S., Eds.), pp. 244-245, Mayflower Scientific Ltd.
[4] Blishchenko, E.Yu., Mernenko, O.A., Yatskin, O.N., Ziganshin, R.H., Philippova, M.M., Karelin, A.A. and Ivanov, V.T. (1996) Biochem. Biophys. Res. Commun. 224, 721–727.
[5] Karelin, A.A., Philippova, M.M. and Ivanov, V.T. (1995) Peptides 16, 693–697.
[6] Zhu, Y.X., Hsi, K.L., Chen, Z.G., Zhang, H.L., Wu, S.X., Zhang, S.Y., Fang, P.F., Guo, S.Y., Kao, Y.S. and Tsou, K. (1986) FEBS Lett. 208, 253–257.
[7] Kim, W.H., Schnaper, W., Nomizu, M., Yamada, Y. and Kleinman, H.K. (1994) Cancer Res. 54, 5005–5010.
[8] Feldman, R.I., Fried, S., Mann, E., Wu, J.M. and Lians, M. (1996) Mol. Pharmacol. 49, 505–514.
[9] Blishchenko, E.Yu., Mernenko, O.A., Mirkina, I.I., Satpaev, D.K., Ivanov, V.S., Tchikin, L.D., Ostrovsky, A.G., Karelin, A.A. and Ivanov, V.T. (1997) Peptides 18, 79–85.
[10] Ivanov, V.T., Karelin, A.A., Philippova, M.M., Nazimov, I.V. and Pletnev, V.Z. (1997) Biopolymers Peptide Sci. 43, in press.
[11] Van de Loosdrecht, A., Ossenkoppele, G. and Beelen, R. (1993) Cancer Res. 53, 4399-4402-4417.
[12] Camby, I., Selmon, I., Bourdel, E., Nagy, N., Danguy, A., Brotchi, J., Pasteels, J.-L., Martinez, J. and Kiss, R. (1996) 30, 130–133.